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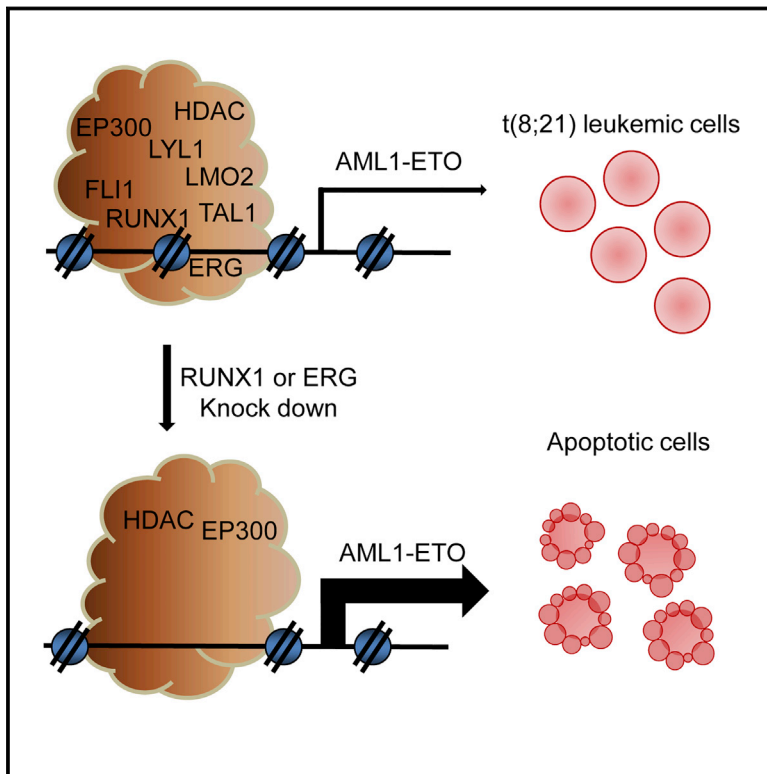
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Cell Reports

The Hematopoietic Transcription Factors RUNX1 and ERG Prevent AML1-ETO Oncogene Overexpression and Onset of the Apoptosis Program in t(8;21) AMLs

Graphical Abstract



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In Brief

As part of the International Human Epigenome Consortium (IHEC), Mandoli et al. investigate the AML1-ETO-associated epigenome, transcriptome, and proteome in t(8;21) patient cells and cell lines. Together their results suggest that a balanced interplay between the chromatin environment and expression of RUNX1 and ERG prevent AML1-ETO oncogene overdose and thereby inhibit apoptosis. Explore the Cell Press IHEC webportal at www.cell.com/consortium/IHEC.

Highlights

- Global analysis of AML1-ETO (AE) in two patient blasts
- The AML1-ETO complex consists of hematopoietic, chromatin, and splicing regulators
- ERG or RUNX1 knockdown upregulates AML1-ETO expression
- AML1-ETO overexpression in differentiated iPSCs induces apoptosis

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The Hematopoietic Transcription Factors RUNX1 and ERG Prevent AML1-ETO Oncogene Overexpression and Onset of the Apoptosis Program in t(8;21) AMLs

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SUMMARY

The t(8;21) acute myeloid leukemia (AML)-associated oncoprotein AML1-ETO disrupts normal hematopoietic differentiation. Here, we have investigated its effects on the transcriptome and epigenome in t(8;21) patient cells. AML1-ETO binding was found at promoter regions of active genes with high levels of histone acetylation but also at distal elements characterized by low acetylation levels and binding of the hematopoietic transcription factors LYL1 and LMO2. In contrast, ERG, FLI1, TAL1, and RUNX1 bind at all AML1-ETO-occupied regulatory regions, including those of the AML1-ETO gene itself, suggesting their involvement in regulating AML1-ETO expression levels. While expression of AML1-ETO in myeloid differentiated induced pluripotent stem cells (iPSCs) induces leukemic characteristics, overexpression increases cell death. We find that expression of wild-type transcription factors RUNX1 and ERG in AML is required to prevent this oncogene overexpression. Together our results show that the interplay of the epigenome and transcription factors prevents apoptosis in t(8;21) AML cells.

INTRODUCTION

The AML1-ETO (RUNX1-RUNX1T1) oncofusion protein, present in 10% of all de novo acute myeloid leukemia (AML) cases, is the result of the translocation t(8;21)(q22;q22), which involves the

AML1 (RUNX1) gene on chromosome 21 and the ETO gene on chromosome 8 (Miyoshi et al., 1991). Expression of the AML1-ETO oncofusion protein in hematopoietic cells results in a stage-specific arrest of maturation and increased cell survival, predisposing cells to develop leukemia (Nimer and Moore, 2004). At the molecular level RUNX1 (Runt-related transcription factor 1; AML1, CBFA2) represents a DNA-binding transcriptional activator factor (Cameron and Neil, 2004; de Bruijn and Speck, 2004), involved in regulation of hematopoiesis and myeloid differentiation, while ETO (eight-twenty-one; MTG8, RUNX1T1) acts as a corepressor by recruiting NCoR/SMRT/HDAC complexes (Davis et al., 2003; Wang et al., 1998). Apart from these repressor interactions, the AML1-ETO fusion protein also assembles into transcription factor complexes. One of these, consisting of AML1-ETO, CBFB, E proteins HEB and E2A, LYL1, LDB1, and LMO2, has recently been suggested to be essential for leukemic maintenance and differentiation block, as the knockdowns of components of this complex delayed leukemogenesis in mice (Sun et al., 2013), stressing the importance of the interplay between the fusion oncogene and other transcription factors.

Mechanistically, the t(8;21) translocation has long been thought to convert the RUNX1 transcriptional activator to a strong repressor by replacing the transactivation domain of RUNX1/AML1 with an almost complete ETO protein, thereby inducing a repressive chromatin environment and gene repression at otherwise activated RUNX1 target sites (Buchi et al., 2014; Meyers et al., 1995; Okuda et al., 1998; Yergeau et al., 1997). Nevertheless, a wild-type copy of RUNX1 is still required to maintain the AML1-ETO leukemic phenotype (Ben-Ami et al., 2013) as knockdown of RUNX1 in t(8;21) leukemia cell lines results in cell death (Hyde et al., 2015).



Over the last years, it has become clear that the repressive nature of AML1-ETO does not define its full biological activities as the fusion protein has also been reported as an activator of transcription (Klampfer et al., 1996; Peterson and Zhang, 2004; Wang et al., 2011). Recent genome-wide studies propose that relative binding of RUNX1 and AML1-ETO at genes determines the final transcriptional outcome (Li et al., 2016), while epigenetically a mechanism for transcriptional activation involving AML1-ETO and p300 interactions has been suggested (Wang et al., 2011). Alternatively, it has been suggested that wild-type RUNX1 interacts with p300, whereas AML1-ETO recruits HDACs, and binding of RUNX1/p300 and AML1-ETO/HDACs is mutually exclusive (Ptasinska et al., 2014). Hence, the precise mechanisms by which AML1-ETO deregulates the RUNX1 program and the histone acetylation machinery remain unclear.

So far the majority of studies have been performed using cell lines with limited validation of findings in clinical samples. In the present study, we investigated AML1-ETO-associated epigenetic modification (i.e., functional changes to the genome that do not involve a change in the DNA) and its relation to gene expression in patient t(8;21) blasts. We explored the AML1-ETO complex and the role of the individual components of this complex in leukemogenesis. We performed genome-wide binding analysis in cell lines and primary blasts, identifying two modules of AML1-ETO action, one on promoter regions and one on enhancer/distal elements. Chromatin immunoprecipitation sequencing (ChIP-seq) studies together with pull-down and mass spectrometry identified differential as well as similar co-binding of regulators of transcription and chromatin modifications in the context of these promoter and distal elements. Using knockdown assays, we show that a balance between AML1-ETO, RUNX1, and ERG expression is required for leukemic maintenance, and that RUNX1 or ERG perturbations result in AML1-ETO overdose and lethality to cells. Together, our results suggest that the balanced interplay of the epigenetic environment and transcription factors retains an anti-apoptotic phenotype in t(8;21) AML cells.

RESULTS

AML1-ETO Binds Promoter and Distal Genomic Elements

To investigate the epigenetic alterations associated with AML1-ETO binding, we used ChIP-seq together with model-based analysis of ChIP-seq (MACS) peak calling (Zhang et al., 2008) to identify 2897 common AML1-ETO peaks in cells of three AML patients with t(8;21) (#186, #12 and #229) (Figure 1A). Subsequently, we profiled six histone modifications (H3K4me3, H3K4me1, H3K27ac, H3K36me3, H3K27me3, and H3K9me3) and accessibility by DNase-seq in AML cells of 2 t(8;21) patients expressing the AML1-ETO gene (Figure S1A). Examining these profiles at the 2897 common AML1-ETO binding sites revealed two distinct profiles for AML1-ETO that are differentially marked by H3K4me3, H3K4me1, and H3K27ac (Figures 1B, 1C, and S1B; Table S1). H3K4me3-enriched AML1-ETO binding sites constitute promoters of active genes (Figures 1D and 1E), as is also apparent from the enrichment

of H3K27ac. In contrast, H3K4me1-enriched regions represent distal elements and open chromatin regions harboring low H3K27ac that are associated (by nearest gene approach) with genes that are lower expressed. Despite low H3K27ac levels, these distal elements did not enrich for the repressive histone modifications H3K27me3 or H3K9me3 (Figure 1F), suggesting that lower gene activity might be related to reduced acetylation levels.

To investigate whether the difference in chromatin composition was related to differential transcription factor binding, we assessed motif enrichment using GimmeMotifs (van Heeringen and Veenstra, 2011). This analysis revealed enrichment for the RUNX1 motif in distal elements, while both are enriched for ETS factor motifs (Figure 1G), in line with previous results showing enrichment of ETS factor motifs and presence of the ETS factors ERG and FLI1 at AML1-ETO binding sites in model cell lines (Martens et al., 2012; Ptasinaka et al., 2012).

To examine which functions are affected by the AML1-ETO fusion product, we assigned promoter and distal-element-associated genes to pathways and calculated enrichment (Figure 1H). This revealed involvement in many signaling pathways, apoptosis, self-renewal, and other functions related to fully differentiated myeloid cells. Interestingly, most pathways were associated with AML1-ETO binding at promoters as well as at distal elements, suggesting that each pathway receives multiple AML1-ETO hits.

Identification of the RUNX1/AML1-ETO Protein Complex

To investigate which proteins might be associated with AML1-ETO binding, we performed pull-down experiments combined with mass spectrometry analysis. As this assay typically requires high cell numbers, we used two cell lines, Kasumi-1 and SKNO-1, which harbor t(8;21) and express AML1-ETO. For pull-down, we used two oligos, one harboring a RUNX1 consensus motif TGTGGT and a control oligo that contains no RUNX1 or other common transcription factor motif (Figure 2A). The RUNX1-containing oligos efficiently pulled down AML1-ETO in Kasumi-1 cells (Figure 2B), while the control shows background binding. Subsequently, we analyzed enrichment for proteins in the RUNX1 oligo pull-down using quantitative mass spectrometry analysis. This revealed specific enrichments in both SKNO-1 and Kasumi-1 cells of proteins associated with the RUNX1-containing oligo, like AML1-ETO, RUNX1, and CBFB (Figures 2C and S2). To identify proteins that are recognized in both RUNX1 pull-downs, we intersected the enriched proteins from both pull-downs and identified 34 common proteins that might be involved in regulation at AML1-ETO sites (Figure 2D; Table S2). Within the common interactors, we identified FUS, a fusion partner of ERG in AML (Sotoca et al., 2015), HDAC1 and HDAC2, deacetylases previously suggested as interactors of AML1-ETO (Wang et al., 1998), several splicing factors as well as LMO2 and LYL1, and two transcription factors previously identified to interact with AML1-ETO (Sun et al., 2013). Interestingly, despite enrichment of the motif in AML1-ETO binding sites (Figure 1G), we did not identify ETS factors in the pull-down, suggesting these might constitute an independent stabilizing factor of the AML1-ETO complex on DNA (Figure 2E).

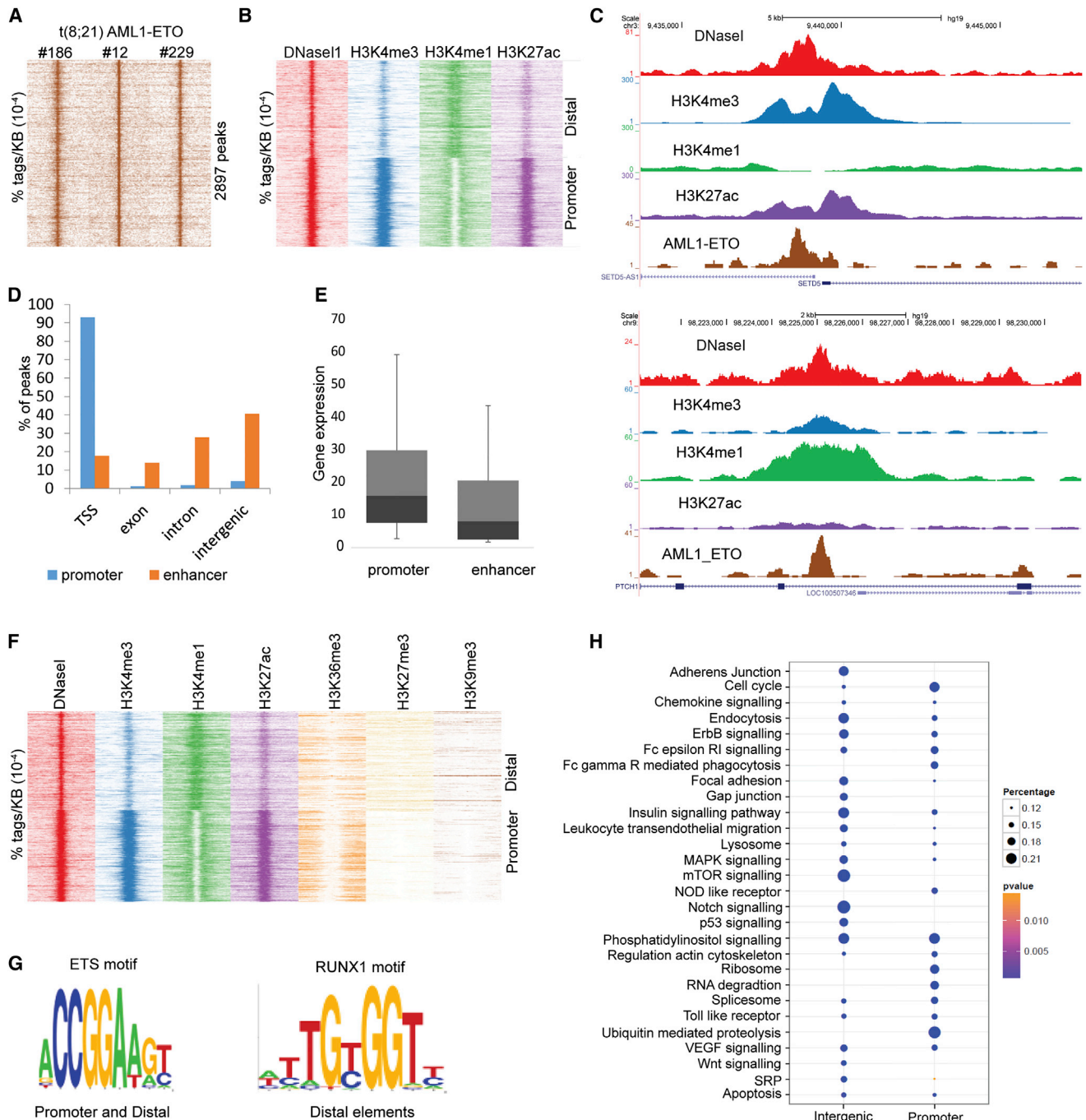


Figure 1. Binding Pattern of AML1-ETO in t(8;21) AMLs

(A) Intensity plot showing the AML1-ETO tag densities in AML patient cells with t(8;21) ($n = 3$) at high confidence AML1-ETO binding sites.

(B) Intensity plot displaying DNaseI accessibility and histone marks at promoter and distal AML1-ETO binding sites in t(8;21) AML #12.

(C) ChIP-seq overview of AML1-ETO binding at the SETD5 promoter and a distal region of PTCH1 in t(8;21) primary AMLs.

(D) Genomic distribution of AML1-ETO binding site locations relative to RefSeq genes. Locations of binding sites are divided in promoter (–500 bp to the transcription start site), exon, intron, and intergenic (everything else).

(E) Expression (reads per kilobase per million reads [RPKM]) of genes associated with AML1-ETO promoter and distal binding sites.

(F) Intensity plot showing active and repressive histone mark enrichment at AML1-ETO binding sites in t(8;21) AML blasts.

(G) Overview of the consensus ETS and RUNX1 motifs found at AML1-ETO binding sites.

(H) Pathway enrichment analysis of genes associated with AML1-ETO binding. Percentage represents the fraction of AML1-ETO-associated genes present in this pathway. SRP, self-renewal pathway.

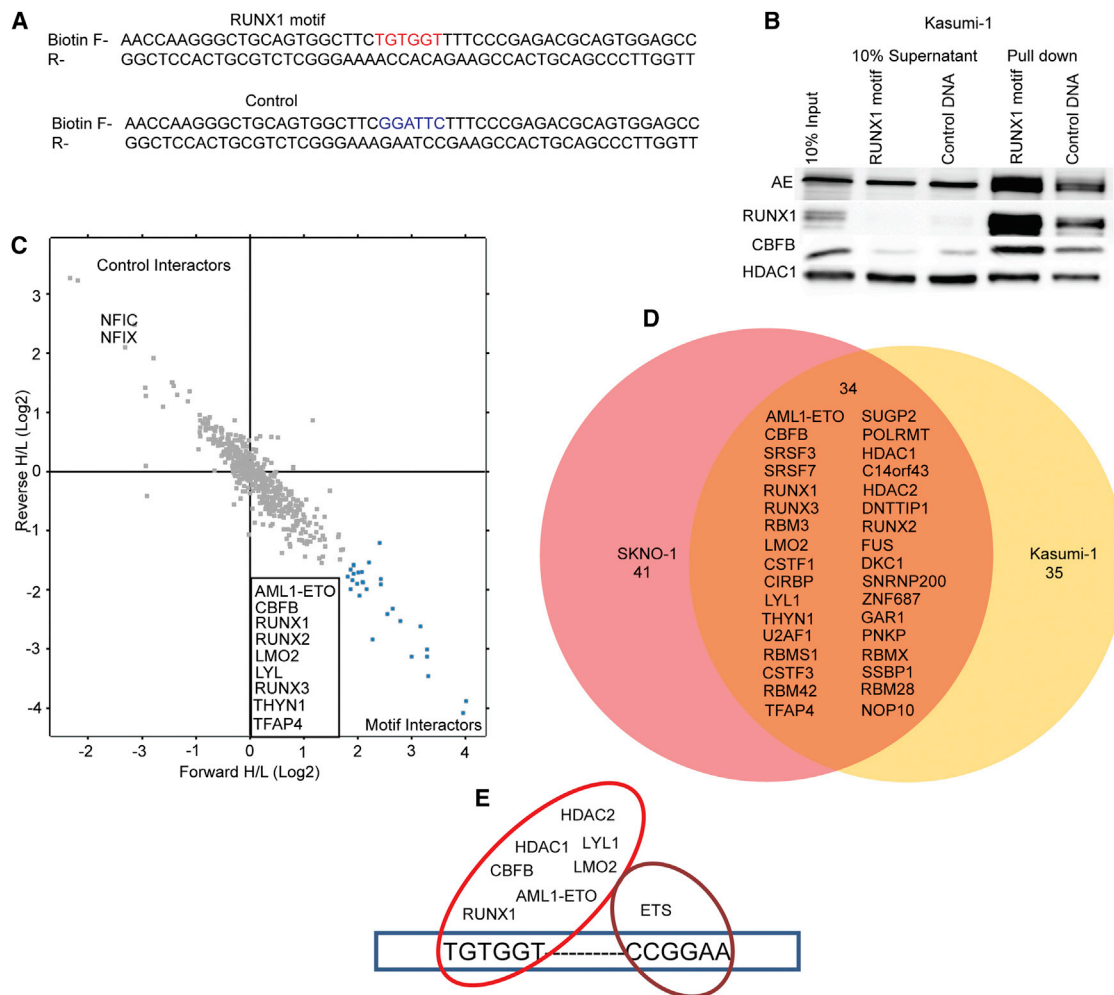


Figure 2. Identification of Interactors of the AML1-ETO/RUNX1 Complex

(A) DNA sequence of oligos used in the pull-down experiment.
(B) Western analysis of identified proteins in the DNA pull-down using the Kasumi-1 cell lysate and AML1-ETO (AE), RUNX1, CBFB, and HDAC1 antibodies.
(C) Scatterplot showing the results of a pull-down mass spectrometry experiment from Kasumi-1 cells. Proteins are plotted by their di-methyl ratios in the forward (x axis) and reverse (y axis) di-methyl experiment. Specific interactors of the AML1-ETO/RUNX1 pull-down are identified in the lower right quadrant.
(D) Venn diagram showing the overlap of 34 proteins identified as RUNX1 motif interactor in pull-down experiments using Kasumi-1 or SKNO-1 cells.
(E) Schematic diagram showing a hypothetical model of ETS factor stabilization of the AML1-ETO protein complex.

Hematopoietic Transcription Factors Bind AML1-ETO-Occupied Genomic Regions

The identification of LMO2 and LYL1 as interactors of AML1-ETO (Figure 2D) (Sun et al., 2013) together with the notion that ETS factor consensus binding sites are enriched at AML1-ETO binding sites suggested that AML1-ETO is present at sites occupied by a heptad of proteins previously identified to be crucial for normal hematopoiesis (Wilson et al., 2010). The genes encoding these transcription factors are commonly expressed in t(8;21) but also in other AMLs (Figure 3A) and not mutated in t(8;21) AMLs (Cancer Genome Atlas Research Network, 2013), suggesting wild-type expression is essential for leukemogenesis. To investigate whether AML1-ETO binding relates to co-occupancy of this heptad of transcription factors, we performed ChIP-seq experiments using antibodies recognizing these fac-

tors in Kasumi-1 t(8;21) cells. We then examined their presence at the previously identified AML1-ETO binding sites in patients AML cells. These results revealed that RUNX1, ERG, FLI1, and TAL1 occupy similar regions as AML1-ETO (Figures 3B and 3C), while GATA2 occupancy was generally low. Co-occupancy of similar genomic regions by RUNX1 and AML1-ETO and ERG and AML1-ETO could be confirmed by re-ChIP experiments (Figure 3D; Martens et al., 2012), suggesting no exclusive allele-specific binding of these factors. Interestingly, in contrast to the other heptad factors, LMO2 and LYL1 binding was enriched at distal elements (Figures 3B and 3C), suggesting that these might be involved in creating a specific chromatin environment (as observed in Figure 1B) at these sites.

Indeed, examining gene responses upon LYL1 knockdown (Sun et al., 2013) revealed that genes associated with AML1-ETO

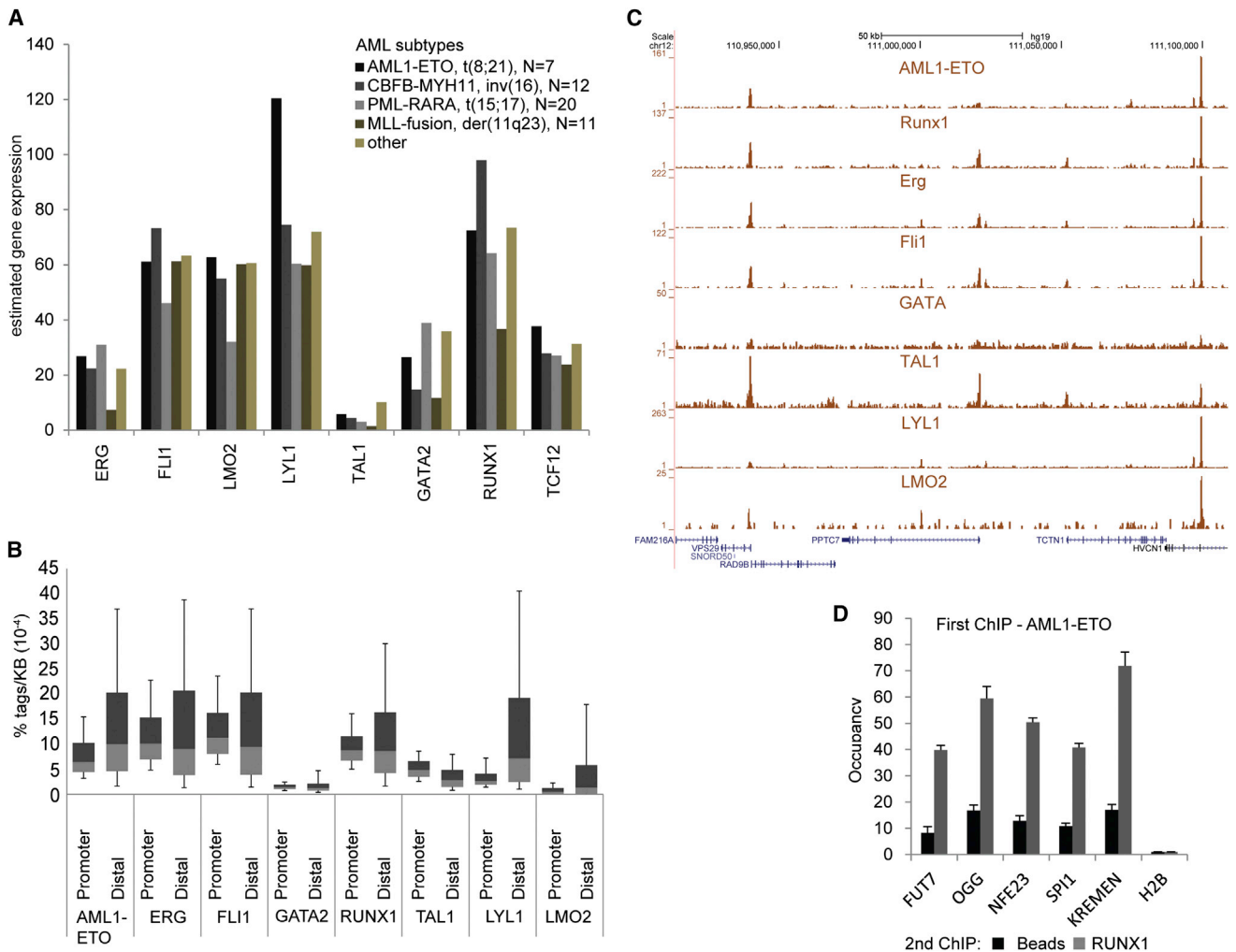


Figure 3. AML1-ETO Co-localizes with Hematopoietic Transcription Factors

(A) Relative expression of hematopoietic transcription factor genes in t(8;21) and other AML subtypes.

(B) Boxplot displaying the tag densities of hematopoietic transcription factors at AML1-ETO binding sites in Kasumi-1 cells. LMO2 and LYL1 are enriched at distal binding sites.

(C) ChIP-seq of AML1-ETO and other transcription factors in Kasumi-1 cells. Overview of the VPS29 and RAD9B AML1-ETO binding sites showing less binding of LMO2 and LYL1 at the VPS29 promoter region, whereas increased binding of LMO2 and LYL1 is observed at the HVCN1 distal region.

(D) Re-ChIP experiment validating AML1-ETO and RUNX1 binding to the same locus. Five binding sites were selected and validated for AML1-ETO/RUNX1 binding by re-ChIP using AML1-ETO in the first round of ChIP followed by a second round using RUNX1 and no antibodies. $n = 3$, mean \pm SD.

distal element binding are increased in expression while promoter-associated genes are less affected (Table S3).

p300 and HDACs Collaborate with AML1-ETO to Regulate Local Histone Acetylation

To examine whether promoter and distal element AML1-ETO-occupied regions have a similar epigenetic pattern in Kasumi-1 cells and patient AML cells, we examined H3 and H4 acetylation at AML1-ETO binding regions in Kasumi-1 cells. This revealed increased acetylation at AML1-ETO promoter sites, while in contrast, lower acetylation at distal regions was observed (Figure 4A), in line with the patient cells (Figure 1B). We identified the deacetylases HDAC1 and HDAC2

as interacting partners of AML1-ETO (Figure 2D), while the acetyltransferase p300 also has been suggested to interact with AML1-ETO (Wang et al., 2011). To examine whether differential occupancy of these enzymes at promoter or distal element regions relates to acetylation levels, we performed ChIP-seq using antibodies against these three proteins. Our results reveal increased occupancy of p300 and HDACs at promoter elements but reduced occupancy at distal elements (Figure 4B). However, within one module the relative occupancy of p300/HDACs is similar (Figure 4B). Re-ChIP experiments revealed the presence of p300/AML1-ETO and HDACs/AML1-ETO at the same locus (Figures 4C and 4D), suggesting that AML1-ETO co-localizes with HDAC/p300

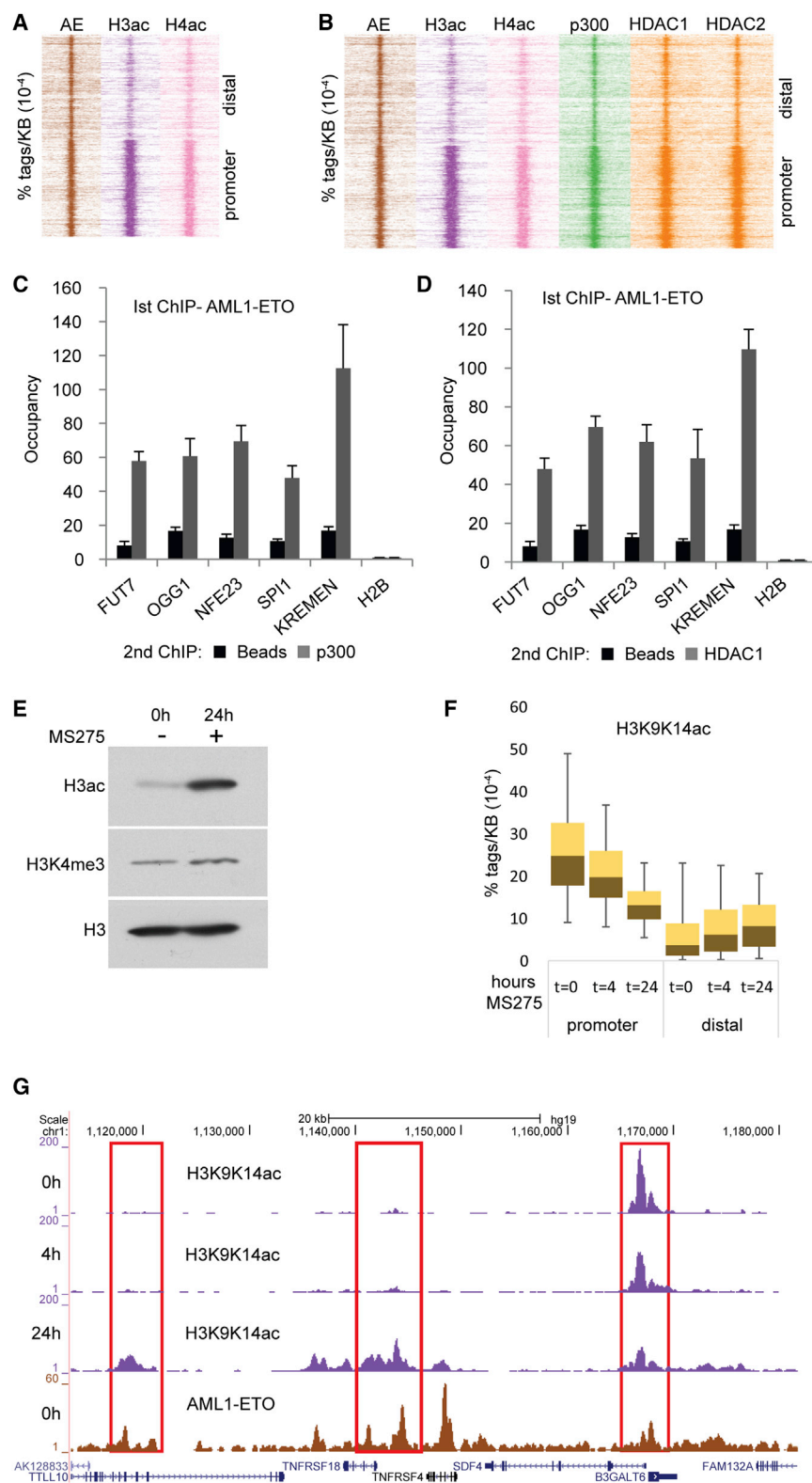


Figure 4. AML1-ETO Distal Binding Regions Are Hypoacetylated

(A) Heatmap displaying H3K9K14ac and H4ac tag densities at promoter and distal binding regions of AML1-ETO (AE). Promoter regions have more acetylation than distal regions.

(B) Intensity plot showing the tag densities of H3ac, H4ac, p300, HDAC1, and HDAC2 at AML1-ETO binding regions. HDACs and p300 are enriched at promoter regions occupied by AML1-ETO.

(C and D) Re-ChIP experiment validating (C) AML1-ETO and p300 binding and (D) AML1-ETO and HDAC1 binding to the same locus. $n = 3$, mean \pm SD.

(E) Western analysis showing increased H3K9K14 acetylation after HDACi treatment in Kasumi-1 cells, whereas H3K4me3 was unaffected.

(F) Boxplot demonstrating decreased H3K9K14ac tag densities at AML1-ETO promoter and increased H3K9K14ac tag densities at distal binding sites after HDACi treatment in Kasumi-1 cells.

(G) Overview of H3K9K14ac acetylation at AML1-ETO binding sites showing increased levels at distal regions and a decrease at the promoter region of SDF4.

complexes, whose counteracting activities (HAT/HDAC) might relate to histone acetylation output.

To see whether this balanced output can be deregulated, we used MS275 (Entinostat), an HDAC inhibitor that induces cell death in Kasumi-1 cells (Duque-Afonso et al., 2011; Nebbioso et al., 2005; Xu et al., 2011). HDACi treatment of Kasumi-1 cells increased H3K9K14 acetylation, whereas H3K4me3 was unaffected (Figure 4E). Examining histone acetylation at AML1-ETO binding sites revealed increased acetylation at distal elements, while at promoter regions a decrease is observed (Figures 4F and 4G) (Dudakov et al., 2013; Ooi et al., 2015; Rafehi et al., 2014). The changes in acetylation level correlate with increased cell death (Figure S3), suggesting a possible involvement of AML1-ETO in regulating the apoptosis program, in line with previous results (Spirin et al., 2014).

t(8;21) AML Addiction to RUNX1 and ERG

Apart from treatment with HDACi, AML1-ETO as well as RUNX1 knockdown have been suggested to induce apoptosis (Ben-Ami et al., 2013; Spirin et al., 2014). To further explore AML1-ETO involvement in regulation of the apoptotic pathway, we examined AML1-ETO binding in patients' AML cells at genes associated with apoptotic programming. Using gene names from KEGG and GO annotation, we identified 172 AML1-ETO binding regions associated with apoptotic genes (Table S4). In t(8;21) AML blast cells, these regions were partially enriched for H3K4me3 and H3K27ac (Figure 5A), representing promoter regions, and partially for H3K4me1 representing distal elements. As indicated above, promoter regions were related to more active genes (Figure 5B), while distal-region-associated genes generally had lower expression. In addition, both promoter and distal regions were enriched for RUNX1 and ETS motifs and bound by ERG, as could be corroborated by ChIP-seq in patient AML cells (Figure 5C). The previously reported dependency on a copy of WT RUNX1 for AML maintenance in t(8;21) AMLs together with the lack of mutations for any of the other heptad factors in t(8;21) AMLs (Cancer Genome Atlas Research Network, 2013) suggests activity of these transcription factors is essential for AML1-ETO leukemogenesis. To investigate whether t(8;21) cells might also be dependent on other transcription factors then RUNX1 for their survival, we chose to focus on ERG, as it was not identified in our RUNX1 proteomic approach (Figure 2D) but still occupied similar genomic regions as AML1-ETO (Figure 3B), suggesting it has a DNA-binding-dependent contribution to the complex. First, we confirmed t(8;21) dependency on RUNX1 by creating a stable inducible RUNX1 knockdown system in Kasumi-1 cells using a specific short hairpin RNA (shRNA) targeting the RUNX1 moiety not present in AML1-ETO (shRUNX1.2) (Figure 5D). Although we only obtained a RUNX1 knockdown of 50% (Figure 5E), cell-cycle analysis using propidium iodide (PI) staining demonstrated a marked increase in the sub-G1 fraction, a hallmark of increased cell death, and a decrease in live cells (Figures 5F and S4A). This increased accumulation of dead cells in sub-G1 was due to apoptosis-mediated cell death as confirmed by Annexin V staining (Figures 5G and S4B). Next, we created a stable knockdown system in Kasumi-1 cells using a shRNA targeting ERG. Similar to RUNX1 knockdown, induction of shRNA expression

(Figures 5H and S4C) resulted in increased apoptosis and decreased cell viability (Figures 5I and S4D–S4G), suggesting expression of ERG is required for leukemic maintenance and addiction to this transcription factor in t(8;21) AML.

To investigate which genes are differentially regulated upon ERG knockdown, we performed RNA-seq using the stable ERG knockdown system in Kasumi-1. This analysis revealed an increased expression of CASP8, CASP6, and CASP10 (Figures 5J and S4H) and decreased expression of BCL2, BIRC7, and BCL2L1, corroborating that the apoptosis program is activated upon knockdown of ERG.

AML1-ETO Overdose Increases Cell Death

Increased AML1-ETO expression has been associated with induction of cell death (Burel et al., 2001; Lu et al., 2006). Interestingly, our RNA-seq results revealed that, upon downregulation of ERG, AML1-ETO expression is increased (Figure S5A). To confirm this observation, we performed reverse transcription quantitative polymerase chain reaction (RT-qPCR) using AML1-ETO-specific primers and western analysis and could again show increased AML1-ETO expression (Figures 6A and S5B). To further examine whether increased AML1-ETO expression correlates with the onset of the apoptosis program, we also examined expression upon RUNX1 knockdown (using shRNA RUNX-1.2). This again revealed increased expression of AML1-ETO by RT-qPCR and western blotting (Figures 6B and 6C).

To investigate whether this increase in AML1-ETO expression is modulated by altered binding of epigenetic modifiers, we performed ChIP using p300 and HDAC1 antibodies. ChIP-qPCR after knockdown of RUNX1 (Figure 6D) or ERG (Figure S5C) revealed increases in p300 and decreases in HDAC1 occupancy at two promoter regions (RUNX1.P1 and RUNX1.P2) of AML1-ETO, suggesting that alterations in transcription factor presence alter epigenetic protein recruitment to the AML1-ETO promoter resulting in increased transcription.

To confirm the effect of AML1-ETO overexpression on cell viability, we used a human induced pluripotent stem cell (iPSC) differentiation system that harbors a dox-inducible AML1-ETO construct, allowing to express the protein at different stages during *in vitro* hematopoietic differentiation. We first tested the differentiation of iPSCs toward the monocytic and granulocytic lineages (Figures 6E and S5D) and confirmed granulocytic differentiation by detecting segmented nuclei and intracellular granules using May-Grunwald and Giemsa staining (Figure 6F, top) and confirmed enrichment of CD15 and/or CD16-positive cells by fluorescence-activated cell sorting (FACS) (Figure S5E). In contrast, we could not detect any segmented nuclei and granules after monocytic differentiation (Figure 6F, bottom) but could observe enrichment for CD14-positive cells (Figure S5F). Using these iPSC cells and expressing AML1-ETO during granulocytic differentiation at levels similar to patient blasts (14 ng/mL dox; Figures 6E and S5G) revealed increased numbers of progenitor (CD34⁺) cells in AML1-ETO-expressing conditions, while lower numbers of granulocytic (CD16-expressing) cells were detected (Figures 6G and 6H), suggesting a differentiation block along this lineage. To further assess the oncogenic potential of our differentiation system, we examined at the molecular level whether AML1-ETO has similar effects on gene expression as in leukemic

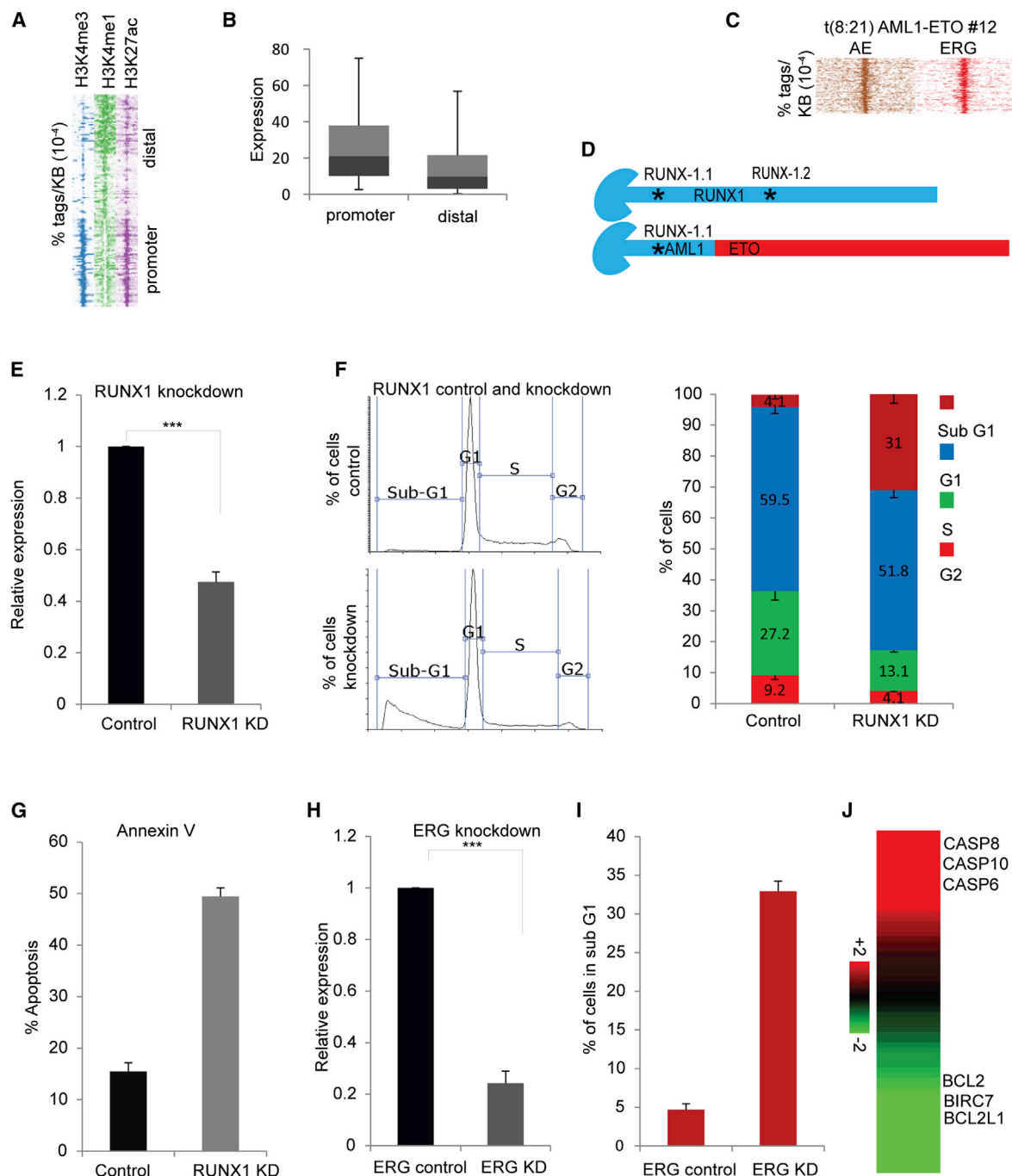


Figure 5. RUNX1 and ERG Are Essential for Kasumi-1 Cells Survival

(A) Heatmap showing H3K4me3, H3K4me1, and H3K27ac tag densities at AML1-ETO binding sites related to genes involved in apoptosis in a t(8:21) AML blast. (B) Expression (RPKM) of apoptotic genes associated with AML1-ETO promoter or distal binding. (C) Intensity plot displaying ERG tag density at AML1-ETO (AE) binding sites related to apoptotic genes in t(8:21) patient blast cells (#12). (D) Schematic diagram showing the shRNA targeting regions for knockdown of either RUNX1 or both RUNX1 and AML1-ETO. *The approximate position of the shRNA. (E) RT-qPCR analysis of RUNX1 before and after induction of shRNA for 72 hr targeting RUNX1 in Kasumi-1 cells. Data are normalized to GAPDH and ***p < 0.001. (F) Cell-cycle analysis by FACS using propidium iodide to measure cellular DNA content in 7 days RUNX1 shRNA control and knockdown cells. Left: cell-cycle analysis. A representative result is shown from one of the four replicate experiments. Right: bar diagram displaying the distribution of the analyzed cells. Data represent the mean ± SD values of four independent experiments. (G) Annexin V staining showing increased apoptosis after KD of RUNX1. n = 3, mean ± SD.

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cells. For this, we selected five genes that are upregulated and two that are downregulated upon AML1-ETO knockdown in AML1-ETO-expressing leukemic cells (Figure S5H, left). This revealed that in our iPSC system AML1-ETO induction has similar effects on both gene sets. For example, genes that are increased in expression upon AML1-ETO knockdown in leukemic cells (such as CD82 and NFE2) show decreased expression upon AML1-ETO induction in the iPSC system (Figure S5H, right). In line with an AML1-ETO oncogene over-dose-inducing apoptosis in Kasumi-1 cells, overexpression (60 ng/mL dox) of AML1-ETO in this system resulted in decreased cell viability (Figures 6E and 6I).

AML1-ETO, RUNX1, and ERG Regulate the Apoptosis Program in t(8;21) AML

Finally, to investigate the relation between an increase of AML1-ETO expression and apoptosis, we generated stable cell lines that allowed double knockdown of RUNX1/AML1-ETO, using an shRNA construct that targets the common RUNX1 part of both (shRUNX1.1) (Figure 5D), or ERG/AML1-ETO, using two independent shRNA constructs. Double knockdown of RUNX1/AML1-ETO or ERG/AML1-ETO resulted in lower expression of both targeted proteins (Figures 7A–7D). Interestingly, in both cases double knockdown rescued the cells from apoptosis (Figures 7E, 7F, and S6A–S6C), with marked decreases in the sub-G1 population. These results suggest that ERG and RUNX1 restrain AML1-ETO from becoming overexpressed and the subsequent activation of an apoptosis program.

DISCUSSION

Leukemic transformation is associated with the dysregulation of the normal cell machinery and characterized by alterations in the epigenome, transcriptome, and proteome. To elucidate the molecular mechanism(s) of AML1-ETO in leukemogenesis, we analyzed gene expression and the epigenome of t(8;21) patient cells and t(8;21) cell lines together with the AML1-ETO/RUNX1 proteome.

We revealed in t(8;21) patient blasts AML1-ETO binding to both promoter and distal elements and that each type of binding is associated with specific chromatin characteristics. Promoter binding sites are high in acetylation and associated with expressed genes, whereas distal sites are reduced in acetylation and linked with lowly expressed genes. Interestingly despite low levels of acetylation, these distal regions are not enriched for other repressive marks, suggesting lower expression is mostly related to reduced acetylation. We did not observe differences in the p300/HDAC balance at promoters and distal regions, although occupancy was generally higher for both p300 and HDACs at promoter regions. These results suggest that additional factors are involved in regulating the acetylation output at AML1-ETO binding sites. Using genome-wide binding

analysis, we demonstrate that at AML1-ETO promoter binding sites occupancy of the transcription factors LMO2 and LYL1 is low, whereas at distal sites increased presence of LMO2 and LYL1 is observed. It is tempting to speculate that differential binding of these factors is involved in repressing acetylation at AML1-ETO distal regions. This would be supported by the observation that knockdown of AML1-ETO results in decreased binding of LMO2 and increased the expression of associated myeloid lineage differentiation genes (Ptasinska et al., 2014).

Our genome-wide analysis also suggests that AML1-ETO and RUNX1 bind genomic regions occupied by both HDACs and p300. However, also mutually exclusive binding of RUNX1/p300 and AML1-ETO/HDACs has been reported (Ptasinska et al., 2014). Our re-ChIP experiments could show co-occupancy of AML1-ETO/RUNX1 as well as AML1-ETO/p300 and AML1-ETO/HDACs at similar genomic locations. Although these findings would be in line with other studies suggesting AML1-ETO/RUNX1 (Li et al., 2016) and AML1-ETO/p300 co-occupancy at single loci (Wang et al., 2011), here, as well as in the other studies, only a limited number of regions were included in the re-ChIP experiment and no genome-wide re-ChIP analysis was performed.

To better understand the activating and repressive nature of AML1-ETO at protein-DNA level, we performed, in addition to ChIP-seq analysis, DNA pull-downs using t(8;21) cell lysates and RUNX1-motif-containing oligos. Apart from previously identified AML1-ETO interactors such as the transcription factors LYL1 and LMO2 and the deacetylases HDAC1 and HDAC2, we also identified several proteins involved in RNA splicing (for example, several RBMs and SRSFs), suggesting that the RNA-processing machinery is directly linked to the transcription regulation machinery and deregulated by AML1-ETO. Interestingly, recent mutational analysis in a large cohort of AMLs identified splicing factors as commonly mutated in AMLs (Cancer Genome Atlas Research Network, 2013), suggesting deregulation of this machinery might be a general feature of AMLs and also involved in t(8;21) leukemogenesis.

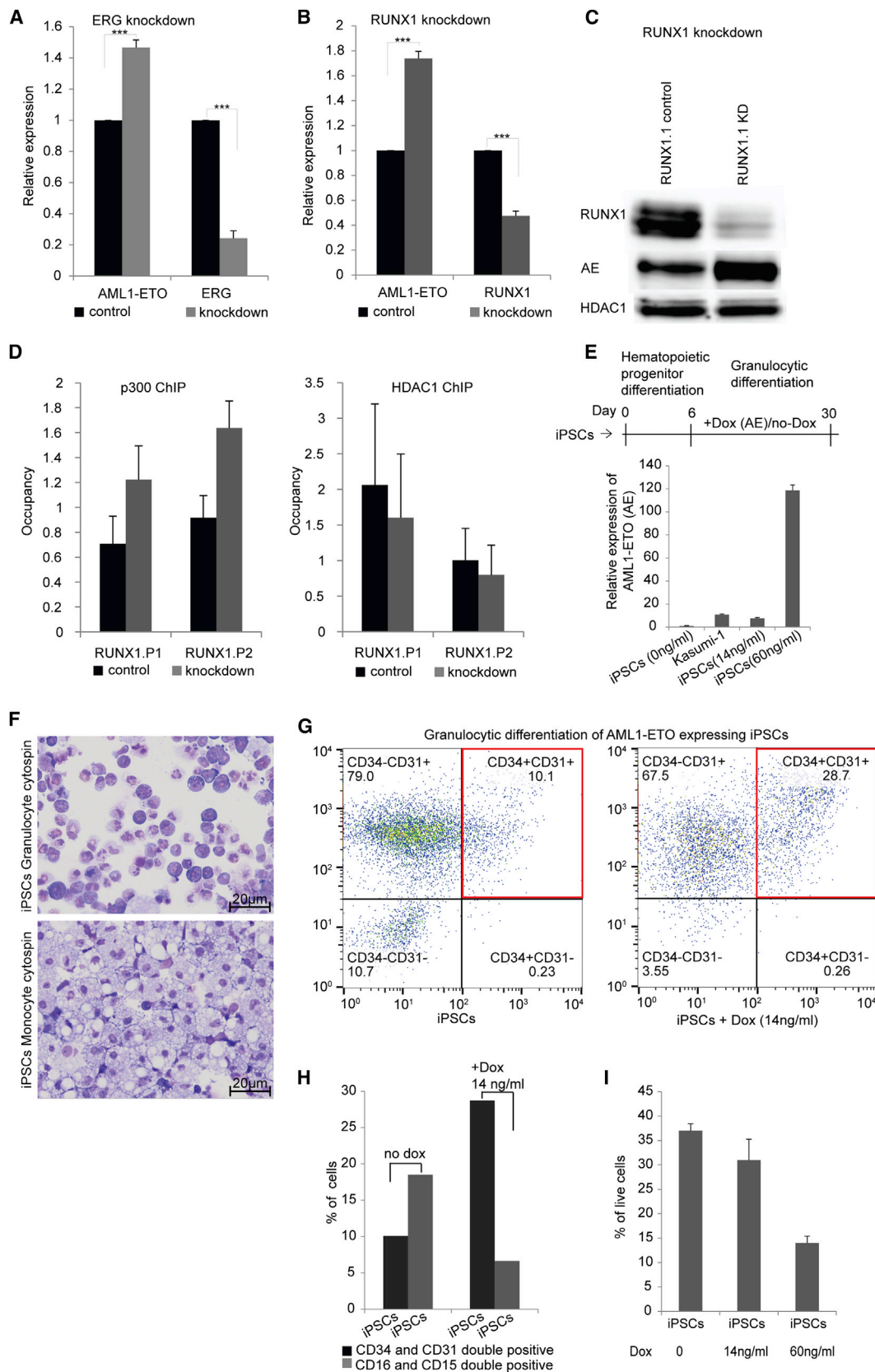
Motif analysis of the patient blasts' AML1-ETO binding regions revealed the presence of ETS motifs, in line with reports that ETS factors are involved in AML1-ETO leukemogenesis (Martens et al., 2012; Trombly et al., 2015). Interestingly, we did not find ETS factors in our RUNX1 motif pull-down analysis suggesting protein-protein interaction is not sufficient to interact with the RUNX1 or AML1-ETO complex, but the additional presence of a DNA consensus binding site is required in order to stabilize interaction with these complexes.

An ETS factor of particular interest is ERG, as it is required for definitive hematopoiesis and self-renewal of hematopoietic stem cells (Loughran et al., 2008; Ng et al., 2011) and showed co-occupancy with AML1-ETO at all binding sites in t(8;21) cell lines and blasts. Moreover, so far no mutation has been reported for ERG in t(8;21) leukemogenesis, underscoring that its activity

(H) RT-qPCR analysis of ERG before and after induction of ERG shRNA expression for 72 hr in Kasumi-1 cells. Data are normalized to GAPDH ***p < 0.001.

(I) Analysis of cells in sub-G1 after 7 days of ERG shRNA or no induction (control). Data represent the mean \pm SD values of four independent experiments. A marked increase was observed in sub-G1 cells in comparison to non-induced cells.

(J) Heatmap showing expression changes of apoptosis-associated genes after ERG knockdown.



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might be essential for AML1-ETO leukemogenesis. Interestingly, upon knockdown of ERG in t(8;21) cells, we observed an increase in sub-G1 cells, indicative of apoptosis onset. In addition, we could corroborate previous reports that upon RUNX1 knockdown a similar phenotype was observed (Ben-Ami et al., 2013) and that a delicate balance of RUNX1 and AML1-ETO is essential for leukemic maintenance of Kasumi-1 cells. Perturbation of this equilibrium by depletion of AML1-ETO leads to loss of self-renewal, whereas knockdown of RUNX1 results in apoptotic cell death (Ben-Ami et al., 2013; Spirin et al., 2014). Here, our ERG knockdown results suggest that apart from RUNX1 and AML1-ETO, t(8;21) cells are also dependent on ERG expression and as such, in addition to RUNX1, are also ERG addicted.

We confirmed the dependency on AML1-ETO/RUNX1 balance by generating a stable inducible RUNX1 knockdown system in Kasumi-1 cells and suggest that increased expression of AML1-ETO might be linked to the induced apoptosis program. The present study demonstrates that not only knockdown of RUNX1, but also knockdown of ERG upregulates AML1-ETO expression and thus interferes with the AML1-ETO/RUNX1 equilibrium, possibly resulting in more AML1-ETO binding to RUNX1 target genes or redistribution of AML1-ETO binding as suggested previously (Ptasinska et al., 2014).

Using an iPSCs model system, we verified that overexpression of AML1-ETO induces cell death, suggesting that only a specific dose of AML1-ETO relates to leukemogenesis, corroborating previous findings (Pabst et al., 2001; Tonks et al., 2004). In addition, the link between oncogene overdose, i.e., higher expression of AML1-ETO, and increased apoptosis, corroborates previous studies of inherent pro-apoptotic activities of AML1-ETO (Lu et al., 2006) and an anti-apoptotic role for RUNX1 (Ben-Ami et al., 2013). Oncogene overdose is a relatively new concept highlighted in particular by recent studies of mutant-BRAF in melanoma and the fusion kinase nucleophosmin–anaplastic lymphoma kinase (NPM-ALK) in anaplastic large cell lymphoma (Amin et al., 2015). To our knowledge, this concept, which is based on mutated kinases, has not been reported for AML-associated oncogenes nor extended to mutated transcription factors.

Importantly, we could show that double knockdown of either RUNX1 and AML1-ETO or ERG and AML1-ETO rescues the cells

from apoptosis, further illustrating the critical role of increased AML1-ETO expression in apoptosis induction and the importance of a finely tuned AML1-ETO/RUNX1 and AML1-ETO/ERG expression equilibrium. Corroborating these results are previous observations in SKNO-1 cells selected for stable continuous ERG knockdown in which reduced AML1-ETO expression (as compared to wild-type SKNO-1 cells) was observed (Martens et al., 2012), which suggested that only subclones that in addition to reduced ERG also harbored low AML1-ETO could survive selection.

Together these results show that a delicate balance of AML1-ETO, RUNX1, and ERG expression is required for leukemic maintenance. Altering this balance might be used as a therapeutic entry point to induce apoptosis in t(8;21) cells.

EXPERIMENTAL PROCEDURES

Cell Culture

Kasumi-1 (Asou et al., 1991) was routinely cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and 1% pen/strep at 37°C. Kasumi-1 shRNA stable cell lines were cultured in tet-free FBS, and shRNA expression was induced for 72 hr for RT-qPCR and 7 days for cell-cycle analysis by adding 600 ng/mL doxycycline. iPSCs cells were generated from megakaryoblast at the Sanquin Research Department of Hematopoiesis, Amsterdam, the Netherlands. Briefly peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation using Ficoll, and then CD34⁺ cells were isolated using CD34 beads. CD34⁺ were initially cultured in CD34 expansion media (Stemspan, 100 µg/SCF, 1 µg/mL interleukin-3 [IL-3], 1 µg/mL IL-6, 1 U/mL TPO, and L4646) for 5 days, and then media was changed to Stemspan medium supplemented with 100 µg/mL SCF, 10 U/mL TPO, and 1 µg/mL IL-1B. On the ninth day, CD34/CD41 cells were sorted and used for iPSCs generation. iPSCs were routinely cultured in E8 Media (Life Technologies).

AML1-ETO-expressing iPSCs cell were generated by using a previously described strategy of knockin using an AAVS1 homology donor vector and CRISPR-Cas9 (Mali et al., 2013). Briefly two million iPSCs were nucleofected with donor vector containing an inducible promoter for expression of the cloned gene (Qian et al., 2014) and a gene targeting vector for the AAVS1 locus (Mali et al., 2013). Transfected cells were plated in one well of a vitronectin (Life Technologies)-coated 6-well plate in E8 media (Life Technologies) supplemented with 10 µM of rock inhibitor for 24 hr. Cells were dissociated using accutase (Life Technologies) and seeded at low density on a vitronectin-coated dish in E8 media together with 0.25 µg/mL puromycin. Cells were selected for puromycin for 14 days, and positive clones were selected by PCR. AML1-ETO iPSCs were routinely maintained in E8 media (Life Technologies) on vitronectin-coated plates. iPSCs generation was performed by

Figure 6. AML1-ETO Oncogene Expression Level Determines the Fate of Leukemic Cells

- (A) RT-qPCR analysis of AML1-ETO and ERG in Kasumi-1 cells, before and after induction of a shRNA targeting ERG for 72 hr. AML1-ETO expression increased after ERG KD. Data are normalized to GAPDH ***p < 0.001.
- (B and C) RT-qPCR (B) and western (C) analysis show increased AML1-ETO (AE) expression after RUNX1 KD for 72 hr in Kasumi-1.
- (D) p300 and HDAC ChIP qPCR before and after induction of RUNX1 shRNA for 72 hr. Two primers were used targeting the promoter of RUNX1/AML1-ETO, and data were normalized with H2B. n = 3, mean ± SD.
- (E) Effect of dox concentration on AML1-ETO expression during granulocytic differentiation of iPSCs. Top: schematic representation of iPSC differentiation protocol toward granulocytes. Bottom: RT-qPCR analysis of AML1-ETO expression during granulocytic differentiation using different dox concentration; data are normalized to GAPDH.
- (F) Cytospin of iPS cells differentiated toward granulocytic and monocytic cells using May-Grunwald and Giemsa staining. Top: granulocyte differentiated cells. Bottom: monocyte differentiated cells.
- (G) Flow cytometry results of iPSC differentiation toward granulocytes with (14 ng/mL dox) or without (no dox) AML1-ETO expression examining CD34/CD31-expressing cells after normal in vitro differentiation (left) or in the presence of AML1-ETO expression (right).
- (H) Bar diagram showing the percentage of CD34 and CD16 cells in AML1-ETO-expressing (+dox 14 ng/mL) and control cells (no dox).
- (I) Effect of increased AML1-ETO expression (using increased concentrations of dox) on viability of iPSCs differentiated along the granulocytic pathway. Cell viability was checked by trypan blue cell counting. n = 3, mean ± SD.

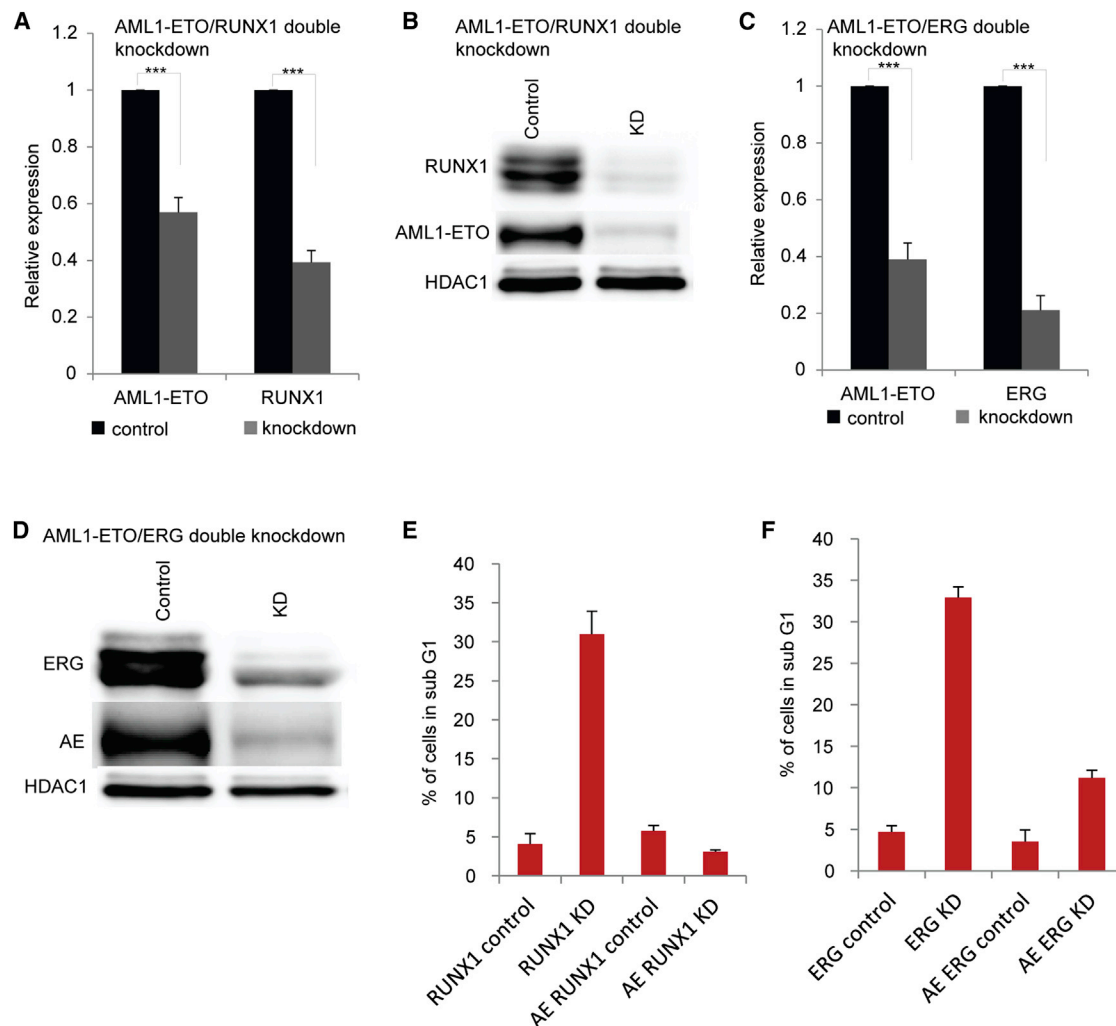


Figure 7. Double Knockdown of AML1-ETO and ERG or AML1-ETO and RUNX1 Rescues Cells from Apoptosis

(A and B) Analysis of double KD of AML1-ETO and RUNX1 by (A) RT-qPCR (***p < 0.001) and (B) western analysis.

(C and D) Double KD of AML1-ETO and ERG is confirmed by (C) RT-qPCR (***p < 0.001) and (D) western analysis.

(E and F) Histograms showing the percentage of sub-G1 cells after (E) AML1-ETO (AE) and RUNX1 or (F) AML1-ETO (AE) and ERG double KD. Data represent the mean \pm SD values of four independent experiments.

Sanquin, Amsterdam. t(8;21) AML blasts from peripheral blood or bone marrow were obtained and processed as previously described (Martens et al., 2012).

Granulocytic and Monocytic Differentiation

For granulocytic and monocytic differentiation, AML1-ETO iPSCs were dissociated using accutase and resuspended in E8 media supplemented with rock inhibitor (10 μ g/mL). Cells were seeded at a density on Geltrex (Life Technologies)-coated 6-well plate, so that only four to five colonies emerge in each well. Cells were maintained in E8 media until individual colonies grew up to approximately 500 μ m in diameter. E8 media was then replaced by stemline media supplemented with 1% penstrep, 1:100 insulin-transferrin-selenium-Ethanolamine (ITS), and cytokines (20 ng/mL BMP4, 40 ng/mL VEGF, and 5 ng/mL bFGF). This day was considered as day 0 of differentiation, and the medium was refreshed after 3 days. On day 6, the cytokines were again replaced with a specific cytokine cocktail for monocyte (50 ng/mL SCF, 50 ng/mL FLT3, 50 ng/mL IL-3, 50 ng/mL M-CSF, and 10 ng/mL TPO) (Niwa et al., 2011) or neutrophil differentiation (50 ng/mL

SCF, 50 ng/mL IL-3, 50 ng/mL G-CSF, and 5 ng/mL TPO) (Morishima et al., 2014), and thereafter medium was changed every 3–4 days. The overall scheme of differentiation is outlined in Figure S5D. Dox (14 ng/mL) was added on the sixth day of differentiation, and cells were kept continuously in dox until analyzed by flow analysis.

ChIP and ChIP-Seq

Chromatin from cell lines was harvested as described (Mandoli et al., 2014). ChIPs were performed using specific antibodies to AML1-ETO (Diagenode, C15310197), RUNX1 (Abcam; ab23980), ERG (Santa Cruz Biotechnology; sc-353), FLI1 (Santa Cruz; sc-356), GATA2 (Santa Cruz; sc-9008), HEB (Santa Cruz; sc-357), and LYL1 (Santa Cruz; sc-374164) and analyzed by quantitative PCR or sequencing analysis. Relative occupancy was calculated as fold over background, for which the second exon of the *Myoglobin* gene or the promoter of the *H2B* gene was used. Chromatin isolation and ChIP-seq from primary t(8;21) AMLs was done according to Blueprint protocols (www.blueprint-epigenome.eu/index.cfm?p=7BF8A4B6-F4FE-861A-2AD57A08D63D0B58) using Diagenode antibodies.

Illumina High-Throughput Sequencing

ChIP-seq libraries for transcription factors were prepared from precipitated DNA of 5 million cells (four to five pooled biological replicas) using the Kapa hyperprep kit. For RNA-seq, 250 ng of RNA was used for ribozero (Illumina MRZ11124) and subsequent library preparation. Libraries were loaded on E-gel and a band corresponding to ~300 bp (DNA + Adaptor) was collected and used for cluster generation on the Illumina HiSeq genome analyzer. The 42- to 50-bp tags were mapped to the reference human genome hg19 using the Burrows-Wheeler Alignment Tool (BWA). For each base pair in the genome, the number of overlapping sequence reads was determined, averaged over a 10-bp window and visualized in the UCSC genome browser (<http://genome.ucsc.edu>). All ChIP-seq and RNA-seq data can be downloaded from the Gene Expression Omnibus (GSE76464 and GSE23730), or the Blueprint DCC (<http://dcc.blueprint-epigenome.eu/#/files>), and the bioinformatic analysis of the data is described in the [Supplemental Information](#).

Pull-Down, Dimethyl Labeling, and Liquid Chromatography Mass Spectrometry Analysis

Nuclear extract preparation and pull-down were performed as described in [Supplemental Experimental Procedures](#). Forward and reverse pull-down proteins were labeled by dimethyl isotopes (Boersema et al., 2009) mixed and measured by Q Exactive mass spectrometer (Thermo Fisher Scientific). Raw data were processed by MaxQuant software, and plots were generated using Perseus.

ACCESSION NUMBERS

The accession numbers for the data reported in this paper are GEO: GSE76464 and GSE23730. Data can also be obtained from the Blueprint DCC (<http://dcc.blueprint-epigenome.eu/#/files>).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.08.082>.

AUTHOR CONTRIBUTIONS

A.M., M.O., R.D., M.T.H., E.T., A.T.J.W., E.M.J.-M., K.B., E.v.d.A., N.S., B.K., F.M., L.N.N., N.C.H., N.A.R., L.A., E.V., and J.H.A.M. designed and performed experiments; A.M., A.A.S., K.H.M.P., M.O., R.D., M.T.H., A.T.J.W., N.C.H., and J.H.A.M. analyzed data; A.M., A.A.S., R.D., K.H.M.P., and J.H.A.M. performed bioinformatic analysis; A.M., A.A.S., L.A., E.V., H.G.S., and J.H.A.M. wrote the paper.

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